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203.* Inhibition of Chitosomal Chitin Synthetase and Growth of *Mucor rouxii* by Nikkomycin Z, Nikkomycin X, and Polyoxin A: A Comparison

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Abstract. The effect of the nucleoside-peptide antibiotics nikkomycin Z, nikkomycin X, and polyoxin A was tested on chitosomal chitin synthetase from yeast cells of the dimorphic fungus *Mucor rouxii*. The K_i was 0.6 μM for polyoxin A and 0.5 μM for nikkomycin X; nikkomycin Z was slightly less inhibitory (K_i = 3.5 μM). Whereas the minimum inhibitory concentrations of the nikkomycins for growth and germination were quite low (about 1 μM, or lower), polyoxin A displayed no antifungal activity against yeast cells and sporangiospores of the test organism, even when present in high concentrations. These results are discussed with respect to structure/activity relationships.

Key words: Fungal chitin synthetase – Inhibitors of chitin synthetase – Nikkomycin Z – Nikkomycin X – Polyoxin A – K_i values – MIC values – *Mucor rouxii*

Nikkomycin, a nucleoside-peptide antibiotic related to the polyoxins (Isono et al. 1969), is a powerful inhibitor of fungal growth (Dähn et al. 1976) as well as of chitin synthetase from *Mucor hiemalis* (ibid.), *Coprinus cinereus* (Brillinger 1979) and the insect *Tribolium castaneum* (Cohen and Casida 1980). Wall-bound (McMurrough et al. 1971) and digitonin-solubilized (Gooday and de Rousset-Hall 1975) chitin synthetase were used in the studies with *M. hiemalis* and *C. cinereus*, respectively. The K_i was 0.5 μM (Brillinger 1979), the minimum inhibitory concentration (MIC) for a number of chitinous fungi about 2–20 μM (Dähn et al. 1976).

Nikkomycin has recently been shown to represent a complex of closely related substances with nikkomycin Z and nikkomycin X as major components (Hagenmaier et al. 1979; König et al. 1980; Fiedler 1981). It is, therefore, not known whether these two contribute equally to the observed growth and chitin synthetase inhibitory effects of the complex. The availability of highly purified nikkomycin Z and nikkomycin X prompted us to solve this problem. *Mucor rouxii* was chosen as the experimental system, for two reasons: (i) growth (Bartnicki-Garcia and Nickerson 1962) as well as chitin synthesis (lit. cit. in Bartnicki-Garcia et al. 1979) are especially well studied, and (ii) it represents the organism of choice to isolate yet another form of chitin synthetase, namely, chitosomes, which are miniorganelles thought to serve as cytoplasmic conveyors of chitin synthetase to its destination at the cell surface (ibid.). We were also interested to compare, in one and the same system, the inhibitory effects of nikkomycins with those of a representative of the polyoxins which, in most cases, display MIC values that are considerably higher than the corresponding K_i's (cf. Gooday 1979).

Materials and Methods

Isolation of Enzyme. Growth of the yeast form of *Mucor rouxii* ATCC 24905, and preparation of purified chitosomes were done as described by Bartnicki-Garcia et al. (1978, 1979). Protein was determined according to Lowry et al. (1951) using bovine serum albumin as the standard.

Chitin Synthetase Assay. The incubation mixture contained, in a total volume of 125 μl, enzyme preparation (50 μl; 9–12 μg protein; $K_{m\,UDP-GlcNAc}$ = 0.8 mM: Furter 1980), Rennilase (a crude acid protease, 125 μg), and (final concentrations of) 0.6 or 1.0 mM UDP-(U-^{14}C)GlcNAc (55,000 dpm), 20 mM GlcNac, and 0.2–6 μM nikkomycin Z, nikkomycin X, or polyoxin A (25 μl of an aqueous solution). As checked by HPLC (Fiedler 1981), the nikkomycins were stable under the experimental conditions used. Incubations were for 10 min at 25° C, reaction rates being linear for at least 30 min. The radioactive chitin was determined by a filtration method (Bartnicki-Garcia et al. 1978).

Determination of MIC Values. To estimate MIC's for yeast cells of *M. rouxii* the organism was grown in a YPG medium under an atmosphere of 30% CO_2/70% N_2 as described by Bartnicki-Garcia et al. (1978), and the culture diluted with nutrient solution to 10^5 cells/ml after an incubation time of 14 h. Portions of 2 ml were incubated for 5 h in varying concentrations of the antibiotics. Growth was monitored turbidometrically (Mallette 1969). The MIC's for germination were assessed by incubating spores (5×10^5 cells/ml; 28° C) in a YPG medium, gassing with CO_2/N_2 (as above) or with air and, after 14 h, selecting those cultures that did not show an increase in OD_{578} in comparison to controls without antibiotics that had been kept at 0° C during the experimental period.

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Abbreviations. MIC, minimum inhibitory concentration (i.e. concentration required to completely suppress growth: cf. Drews, 1979); GlcNAc, N-acetyl-D-glucosamine; UDP-GlcNAc, uridine 5′-diphospho-N-acetyl-D-glucosamine

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Chemicals. UDP-N-acetyl-(U-^{14}C)glucosamine (213 Ci/mol) was purchased from Radiochemical Centre Amersham. Rennilase was a gift of Schweizerische Ferment AG, Basel, Switzerland. The nikkomycins were isolated by J. Delzer and R. Kurth, University of Tübingen, and polyoxin was donated by Drs. I. Frosch, University of Tübingen, and W. Keller-Schierlein, Swiss Federal Institute of Technology, Zürich.

Results and Discussion

All antibiotics tested strongly inhibited chitosomal chitin synthetase, the K_i values being about 3.5 µM for nikkomycin Z, 0.5 µM for nikkomycin X, and 0.6 µM for polyoxin A. The kinetic data obtained plotted according to Dixon (1953) indicated inhibition of a competitive type (Figs. 1 and 2). Whereas the nikkomycins had also a very high capacity to inhibit growth and germination, polyoxin A, on the other hand, did not display any in vivo effect in the test organism, even when present in a concentration as high as 100 µM (Table 1). The MIC's of the nikkomycins for growth were about the same as the K_i's, thus supporting the contention that the antifungal action of these compounds results from their interaction with chitin synthetase only, i.e. does not involve an additional target. Germination appears to be somewhat more affected by the nikkomycins than growth.

The results presented furthermore demonstrate that the chitin synthetase and growth inhibitory effects of nikkomycin described earlier (Dähn et al. 1976; Brillinger 1979) are not due to a single chemical entity of the complex: nikkomycin Z and nikkomycin X are both active, in vitro as well as in vivo. The K_i value of chitin synthetase for nikkomycin X being about 7 times lower than that of nikkomycin Z probably reflects a genuine difference as also in vivo the former is somewhat more inhibitory. It seems that certain functional groups as substituents at the nucleoside base (see Fig. 3) are involved in modifying the chitin synthetase inhibitory efficiency of the nikkomycins (as well as of the polyoxins: cf. Hori et al. 1971). The inhibitor constant of chitosomal chitin synthetase for polyoxin A is, thus, identical to that of chitin synthetase present in a plasmalemma fraction isolated from yeast spheroplasts (Keller and Cabib 1971). The K_i observed here for polyoxin A and nikkomycin X is, furthermore, also the same as reported for polyoxin D in a crude particulate chitin synthetase preparation from *M. rouxii* (Bartnicki-Garcia and Lippman 1972). The fact that the nikkomycins and the polyoxins inhibit the enzyme by the same mode of action and also are equally effective in doing so (Figs. 1 and 2) finally opens up the possibility nikkomycins to replace polyoxin D which has been widely used as a tool to study chitin synthesis but is no longer available to most workers in the field.

The MIC values of the three chitin synthetase inhibitors studied were remarkably different (Table 1). In contrast to polyoxin A, the nikkomycins proved to be excellent in suppressing growth and germination. Their inhibitory capacity might be even higher than that of the polyene macrolide antibiotics and well-known clinically used fungicides amphotericin B, nystatin and pimaricin (Rast and Bartnicki-Garcia 1981). In not being affected even by high concentrations of polyoxin A the yeast cells of dimorphic *M. rouxii* behave as the true yeasts which are reported to be insensitive to polyoxins (cf. Misato and Kakiki 1977). The fundamental difference observed in the antifungal capacity of

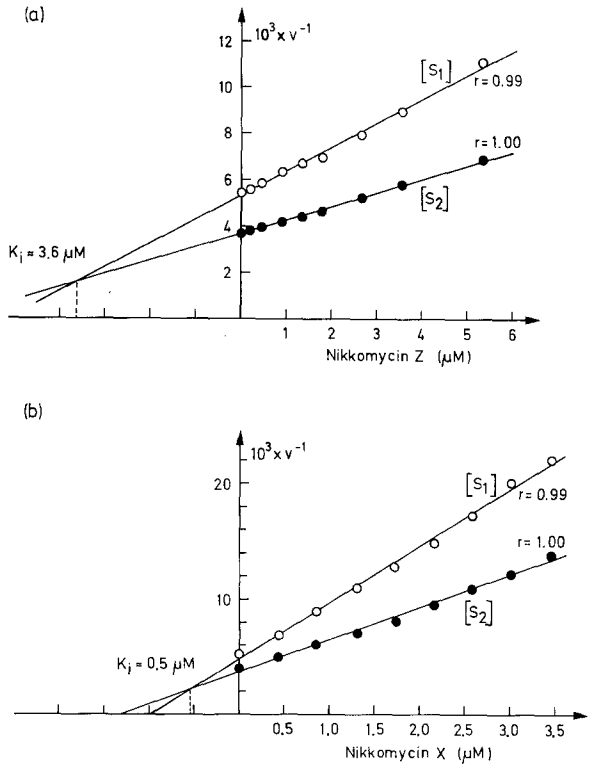

Fig. 1. Kinetics of inhibition of chitosomal chitin synthetase by (**a**) nikkomycin Z and (**b**) nikkomycin X (Dixon plots of activity). [S_1] and [S_2], substrate concentrations used, 0.6 and 1.0 mM UDP-GlcNAc, respectively; v, incorporation rate of substrate into chitin (nmol/min · mg protein)

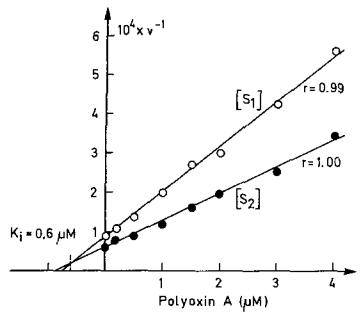

Fig. 2. Kinetics of inhibition of chitosomal chitin synthetase by polyoxin A (Dixon plot of activity). For details, see caption to Fig. 1

Table 1. Inhibition of growth and germination in *Mucor rouxii* by nucleoside-peptide antibiotics: MIC values

Antibiotic tested	Minimum inhibitory concentration (µM)		
	Yeast cells	Sporangiospores	
		aerobic	anaerobic
Nikkomycin Z	1.7	1.7	< 0.5
Nikkomycin X	1.3	< 0.5	< 0.5
Polyoxin A	> 100[a]	> 100[a]	> 100[a]

[a] Highest concentration tested

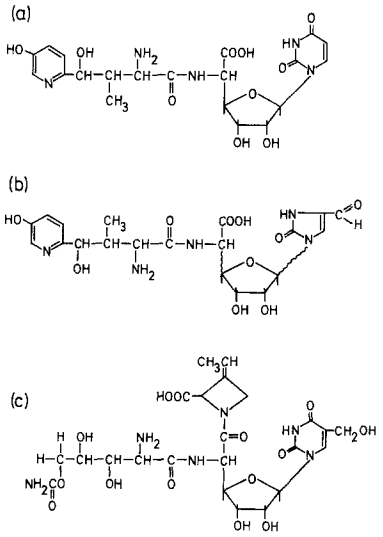

Fig. 3. Structural formulae of nikkomycin Z (**a**), nikkomycin X (**b**), and polyoxin A (**c**)

polyoxin A and the nikkomycins tested could not be explained simply by assuming that the former, being a modified tripeptide (see Fig. 3), would not be able to enter the cell by the (hypothetical) carrier system supposedly used by those polyoxins that can be considered as substituted dipeptides (Gooday 1979), for the following considerations: (i) the modified dipeptide polyoxin D (Isono et al. 1969), displaying a K_i of about 0.5 μM with crude as well as with chitosomal chitin synthetase (Bartnicki-Garcia and Lippman 1972, Ruiz-Herrera et al. 1977), also has a very high MIC value (about 200 – 500 μM; obtained in basically the same system as used in the present work: Bartnicki-Garcia and Lippman 1972), and (ii) *M. hiemalis* is equally sensitive to dipeptide and tripeptide nikkomycins (Fiedler 1981). The MIC of nikkomycin I, a substituted tripeptide (Hagenmaier et al. 1981), for *M. rouxii* yeast cells is, however, similar to that of polyoxin A in the same test system (Müller, unpublished data), an observation supporting the contention of the importance of the carrier system involved (see above) in the uptake of nikkomycins for their antifungal activity. Thus, the reason for the excellent in vivo efficiency of the nikkomycins tested as compared to that of the polyoxins in general (lit. cit. in Gooday 1979; note that merely inhibitory concentrations are given, not the more exacting MIC's) remains to be established. The fact as such does, however, represent a good prospect for the future use of nikkomycins as agricultural fungicides.

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